

## *Escherichia coli* endonuclease III is not an endonuclease but a $\beta$ -elimination catalyst

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The oligonucleotide  $[5'-^{32}\text{P}]\text{pdT}_8\text{d}(-)\text{dT}_n$ , containing an apurinic/apyrimidinic (AP) site  $[\text{d}(-)]$ , yields three radioactive products when incubated at alkaline pH: two of them, forming a doublet approximately at the level of  $\text{pdT}_8\text{dA}$  when analysed by polyacrylamide-gel electrophoresis, are the result of the  $\beta$ -elimination reaction, whereas the third is  $\text{pdT}_8\text{p}$  resulting from  $\beta\delta$ -elimination. The incubation of  $[5'-^{32}\text{P}]\text{pdT}_8\text{d}(-)\text{dT}_n$ , hybridized with poly(dA), with *E. coli* endonuclease III yields two radioactive products which have the same electrophoretic behaviour as the doublet obtained by alkaline  $\beta$ -elimination. The oligonucleotide  $\text{pdT}_8\text{d}(-)$  is degraded by the 3'-5' exonuclease activity of T4 DNA polymerase as well as  $\text{pdT}_8\text{dA}$ , showing that a base-free deoxyribose at the 3' end is not an obstacle for this activity. The radioactive products from  $[5'-^{32}\text{P}]\text{pdT}_8\text{d}(-)\text{dT}_n$  cleaved by alkaline  $\beta$ -elimination or by *E. coli* endonuclease III are not degraded by the 3'-5' exonuclease activity of T4 DNA polymerase. When DNA containing AP sites labelled with  $^{32}\text{P}$  5' to the base-free deoxyribose labelled with  $^3\text{H}$  in the 1' and 2' positions is degraded by *E. coli* endonuclease VI (exonuclease III) and snake venom phosphodiesterase, the two radionuclides are found exclusively in deoxyribose 5-phosphate and the  $^3\text{H}/^{32}\text{P}$  ratio in this sugar phosphate is the same as in the substrate DNA. When DNA containing these doubly-labelled AP sites is degraded by alkaline treatment or with Lys-Trp-Lys, followed by *E. coli* endonuclease VI (exonuclease III), some  $^3\text{H}$  is found in a volatile compound (probably  $^3\text{H}_2\text{O}$ ) whereas the  $^3\text{H}/^{32}\text{P}$  ratio is decreased in the resulting sugar phosphate which has a chromatographic behaviour different from that of deoxyribose 5-phosphate. Treatment of the DNA containing doubly-labelled AP sites with *E. coli* endonuclease III, then with *E. coli* endonuclease VI (exonuclease III), also results in the loss of  $^3\text{H}$  and the formation of a sugar phosphate with a lower  $^3\text{H}/^{32}\text{P}$  ratio that behaves chromatographically as the  $\beta$ -elimination product digested with *E. coli* endonuclease VI (exonuclease III). From these data, we conclude that *E. coli* endonuclease III cleaves the phosphodiester bond 3' to the AP site, but that the cleavage is not a hydrolysis leaving a base-free deoxyribose at the 3' end as it has been so far assumed. The cleavage might be the result of a  $\beta$ -elimination analogous to the one produced by an alkaline pH or Lys-Trp-Lys. Thus it would seem that *E. coli* 'endonuclease III' is, after all, not an endonuclease.

### INTRODUCTION

Endonuclease III is a basic protein (pI 10) which introduces chain scission in u.v.-irradiated DNA; it is active in the presence of EDTA (Radman, 1976). Endonuclease III is also active on X-irradiated,  $\text{OsO}_4$ -treated, or depurinated DNA; the strand scissions leave 5'-phosphate ends (Gates & Linn, 1977). The target of endonuclease III is a thymine with a saturated 5,6 double bond; the enzyme is a DNA glycosylase that releases the modified thymine and also a 3' AP endonuclease that cuts the phosphodiester bond 3' to the AP site leaving a 3'-terminal deoxyribose that cannot be utilized by DNA polymerase I (Demple & Linn, 1980; Warner *et al.*, 1980).

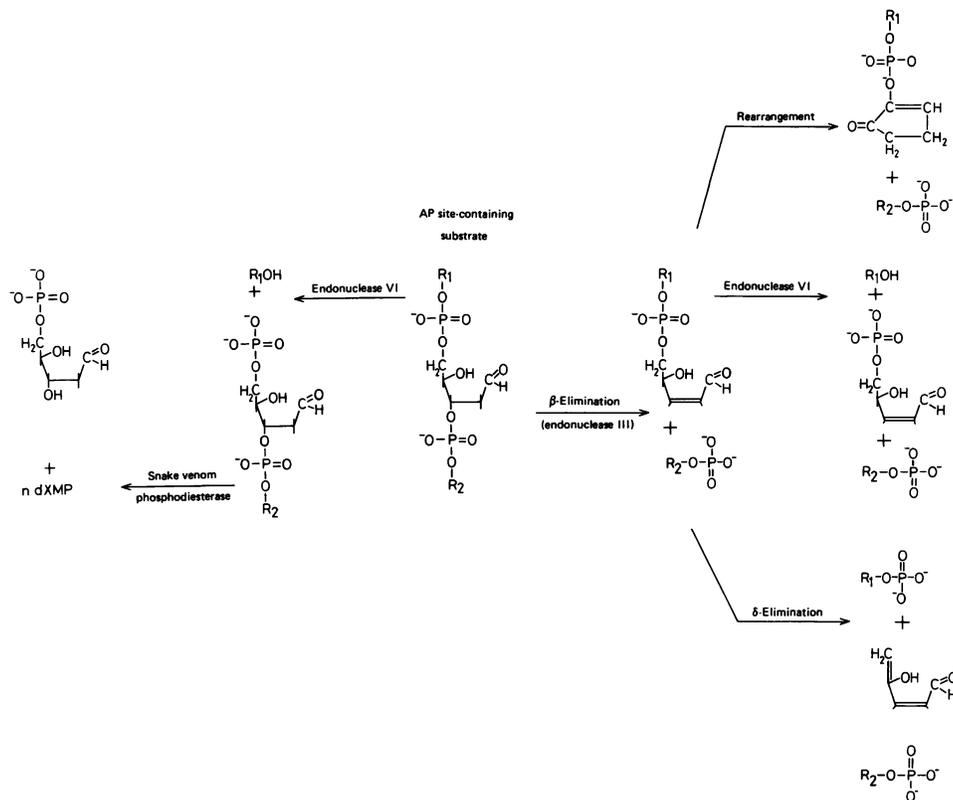
Katcher & Wallace (1983) and Breimer & Lindahl (1984) have completely purified endonuclease III. It has an  $M_r$  of 25000 and does not need any divalent cation to be active. It is a DNA glycosylase that excises various thymine oxidation products like thymine glycol, 5-

hydroxy-5-methylhydantoin, methyltartronylurea and urea; it is also a 3' AP endonuclease that leaves 5'-phosphate ends (see also Doeberl *et al.*, 1986).

Thinking that the nicking 3' to the AP site is necessarily the result of the hydrolysis of the phosphodiester bond, all these authors suppose that it leaves a 3' base-free deoxyribose although they do not present evidence for the nature of the 3' end; the assumption of the presence of a 3'-OH end (see, for instance, Katcher & Wallace, 1983) is made in spite of the observation that the nicked DNA is not a substrate for DNA polymerase I, and that, for the repair to proceed, the 3' end must first be activated by treatment with *E. coli* endonuclease VI or endonuclease IV.

In a preceding paper (Bailly & Verly, 1984), we have shown that a 3'-terminal base-free deoxyribose is not an obstacle to the 3'-5' exonuclease activity of the Klenow fragment of *E. coli* DNA polymerase I. It thus seemed that the nicking by endonuclease III 3' to the AP site could not be the result of the hydrolysis of the

Abbreviations used: AP, apurinic or apyrimidinic; d(-), symbol for base-free deoxyribose (AP site);  $\text{pdT}_8$ , octonucleotide constituted of thymidine 5'-phosphate residues;  $[5'-^{32}\text{P}]\text{pdT}_8$ ,  $\text{pdT}_8$  labelled with  $^{32}\text{P}$  in the terminal 5'-phosphate.



**Fig. 1. Degradation of an AP-site containing oligodeoxynucleotide**

Hydrolysis by *E. coli* endonuclease VI (exonuclease III) and snake venom phosphodiesterase gives deoxyribose-5-phosphate (left).  $\beta$ -Elimination leads to the loss of  $H^+$  from the 2' position of the base-free deoxyribose; when followed by *E. coli* endonuclease VI (exonuclease III) hydrolysis, it gives a 2',3'-unsaturated derivative of deoxyribose 5-phosphate (right). The  $\beta$ -elimination product can also undergo a rearrangement (Jones *et al.*, 1968) and/or a  $\delta$ -elimination.

phosphodiester bond leaving 3'-OH and 5'-phosphate ends. In the present paper, we present evidence suggesting that the mechanism of the 3' nicking is rather a  $\beta$ -elimination (Fig. 1).

## MATERIALS AND METHODS

All the manipulations were carried out under sterile conditions.

### Preparation of *E. coli* endonuclease III

Endonuclease III was prepared from *Escherichia coli* BW 420, an over-producer containing a *nth* gene (Cunningham & Weiss, 1985) in multicopy ColE1 plasmid, that was kindly sent to us by B. Weiss and cultivated in the presence of colicin E1 as recommended. The purification was carried out up to fraction IV as described by Breimer & Lindahl (1984); the enzyme was followed by its nicking activity, destroyed by 5 min of heating at 60 °C, on alkylated depurinated [ $^3H$ ]DNA (Paquette *et al.*, 1972). The purified enzyme was kept at -20 °C in 15 mM-potassium phosphate, pH 7.4, containing 0.5 M-NaCl, 1 mM-EDTA, 7 mM-2-mercaptoethanol and 5% (v/v) glycerol.

The enzyme preparation nicked  $OsO_4$ -treated RF-I [ $^3H$ ]DNA from coliphage  $\phi$ X174. In the presence of EDTA, it was without any non-specific nuclease activity on double-stranded or single-stranded DNA.

### Other enzymes

Endonuclease IV was prepared from *E. coli* B41 according to Ljungquist (1977); the purification was stopped after the DNA-cellulose chromatography. The enzyme was kept at -20 °C in 10 mM-Tris/HCl, pH 8.0, containing 0.5 M-NaCl and 50% (v/v) glycerol.

Uracil-DNA glycosylase was prepared from *E. coli* B41 according to Lindahl *et al.* (1977). The enzyme was kept at -20 °C in 30 mM-Tris/HCl, pH 7.4, containing 0.4 M-NaCl, 1 mM-EDTA, 1 mM-dithiothreitol and 5% (v/v) glycerol.

Snake venom phosphodiesterase (from *Crotalus atrox*, type IV, Sigma) was purified by Blue-Sepharose chromatography according to Oka *et al.* (1978) to eliminate contaminating phosphatases.

Coliphage T4 DNA polymerase and coliphage T4 polynucleotide 5'-OH kinase were from P-L Biochemicals, *Aspergillus oryzae* S1 nuclease and calf-intestine alkaline phosphatase (VII S) were from Sigma, *E. coli* endonuclease VI (exonuclease III) and calf-thymus deoxynucleotidyl terminal transferase were from BRL, and *E. coli* endonuclease-free DNA polymerase I was from Boehringer.

### [5'- $^{32}P$ ]pdT<sub>8</sub>dA

pdT<sub>8</sub>dA (P-L Biochemicals; 8.84 nmol) in 150  $\mu$ l of 50 mM-Tris/HCl, pH 8.5, containing 10 mM-MgCl<sub>2</sub> and 0.1 mM-ZnCl<sub>2</sub>, was incubated with calf-intestine alkaline

phosphatase (2.4 units) for 1 h at 37 °C. After deproteinization with chloroform/isoamyl alcohol (24.1; v/v), the aqueous phase was dried. The residue was redissolved in 150  $\mu$ l of 270  $\mu$ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (3 Ci/mmol), containing 2 mM-dithiothreitol and, after addition of T4 polynucleotide kinase (30 units in 3  $\mu$ l), the mixture was incubated for 1 h at 37 °C; the reaction was stopped by addition of 20  $\mu$ l of 0.2 M-EDTA, pH 9.0.

$[\text{5}'\text{-}^{32}\text{P}]\text{pdT}_8\text{dA}$  was deproteinized and desalted by chromatography on a NENSORB cartridge (New England Nuclear); it was collected in 600  $\mu$ l of 20% (v/v) ethanol. After solvent evaporation, the sample was dissolved in 100  $\mu$ l of stop solution (90% formamide in 0.1 M-Tris/borate, pH 8.3, containing 2 mM-EDTA, 0.02% Bromophenol Blue and 0.02% Xylene Cyanol) and submitted to preparative gel electrophoresis. The  $[\text{5}'\text{-}^{32}\text{P}]\text{pdT}_8\text{dA}$  band was localized by autoradiography and the excised band was eluted twice with 3 ml of 0.1 M-Tris/HCl, pH 7.5, containing 1 mM-EDTA. The mixed eluate was chromatographed on NENSORB to yield the purified  $[\text{5}'\text{-}^{32}\text{P}]\text{pdT}_8\text{dA}$  (3.9 nmol; 2  $\mu$ Ci/nmol) in 600  $\mu$ l of 20% (v/v) ethanol.

#### Oligo(dT)-extended $[\text{5}'\text{-}^{32}\text{P}]\text{pdT}_8\text{dA}$

$[\text{5}'\text{-}^{32}\text{P}]\text{pdT}_8\text{dA}$  (1.3 nmol; 2  $\mu$ Ci/nmol), dTTP (1 mM) and deoxynucleotidyl terminal transferase (150 units) in 400  $\mu$ l of 100 mM-potassium cacodylate, pH 7.2, containing 2 mM- $\text{CoCl}_2$ , 0.2 mM-dithiothreitol and bovine serum albumin (1 mg/ml), were incubated at 37 °C for 16 h. The  $[\text{5}'\text{-}^{32}\text{P}]\text{pdT}_8\text{dAdT}_n$  so obtained was purified by poly(A)-Sephacryl 4B chromatography keeping only the polymers with more than 15 nucleotides as checked on a sample by polyacrylamide-gel electrophoresis followed by autoradiography. After desalting by NENSORB chromatography, the  $[\text{5}'\text{-}^{32}\text{P}]\text{pdT}_8\text{dAdT}_n$  mixture (1.2 nmol; 2  $\mu$ Ci/nmol) was collected in 600  $\mu$ l of 20% (v/v) ethanol.

#### Acid depurination

A part of the  $[\text{5}'\text{-}^{32}\text{P}]\text{pdT}_8\text{dA}$  was completely depurinated by heating for 1 h at 65 °C in 10 mM-HCl/1 mM-EDTA; the depurinated oligonucleotide will be called  $[\text{5}'\text{-}^{32}\text{P}]\text{pdT}_8\text{d}(-)$ .

The same conditions applied to  $[\text{5}'\text{-}^{32}\text{P}]\text{pdT}_8\text{dAdT}_n$  induced partial degradation of the depurinated oligonucleotide by  $\beta$ -elimination.  $[\text{5}'\text{-}^{32}\text{P}]\text{pdT}_8\text{dAdT}_n$  was completely depurinated by incubation for 24 h at 37 °C in 30 mM-HCl/1 mM-EDTA (Kochetkov & Budovskii, 1972). The depurinated oligonucleotide will be called  $[\text{5}'\text{-}^{32}\text{P}]\text{pdT}_8\text{d}(-)\text{dT}_n$ .

#### Polyacrylamide-gel electrophoresis

Denaturing gels (20% polyacrylamide) were prepared from 60 g of urea, 60 ml of 38% acrylamide/2% bisacrylamide, 12 ml of 1 M-Tris/borate, pH 8.3, 20 mM-EDTA, 4 ml of water, 800  $\mu$ l of 10% (w/v) ammonium persulphate and 36  $\mu$ l of tetramethylethylenediamine. The 33 cm  $\times$  40 cm gels had a 0.8 mm thickness. The oligonucleotides to be analysed were dried, dissolved in stop solution, and 10  $\mu$ l samples were placed in the wells (12 mm  $\times$  0.8 mm).

#### Elimination of poly(dA) from oligo(dT)-poly(dA) duplexes

The sample (100  $\mu$ l), after heating for 5 min at 50 °C, was mixed with a cold (0 °C) suspension (500  $\mu$ l) of

poly(A)-Sephacryl 4B (Pharmacia) (50%) in 50 mM-Tris/HCl, pH 7.0, containing 50 mM-NaCl, and 1 mM EDTA, and the mixture was kept on ice. After sedimentation, the supernatant was discarded and the resin was washed twice with 500  $\mu$ l of 0.1 M- $\text{NH}_4\text{HCO}_3$ . The resin, again suspended in 500  $\mu$ l of 0.1 M- $\text{NH}_4\text{HCO}_3$ , was heated for 10 min at 50 °C; after sedimentation, the supernatant was recovered and lyophilized.

#### Preparation of $[\alpha\text{-}^{32}\text{P}]\text{dUTP}$ and $[\text{1}'\text{,2}'\text{,5-}^3\text{H}]\text{dUTP}$

A mixture of  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$  (250  $\mu$ Ci; 410 Ci/mmol) and  $[\text{1}'\text{,2}'\text{,5-}^3\text{H}]\text{dCTP}$  (500  $\mu$ Ci; 62 Ci/mmol) (both from Amersham) in 160  $\mu$ l of 1 M-NaOH was deaminated by heating for 18 h at 70 °C (Clements *et al.*, 1978) before being neutralized with 200  $\mu$ l of 1 M-Tris/HCl, pH 1.0. A chromatographic analysis of an aliquot on poly(ethylene imine)-cellulose indicated that 35% of the radioactivity ( $^{32}\text{P}$  or  $^3\text{H}$ ) was in dUTP, 8% in dCTP, the rest being in monophosphates and diphosphates.

#### DNA with $^{32}\text{P}$ - and $^3\text{H}$ -labelled AP sites

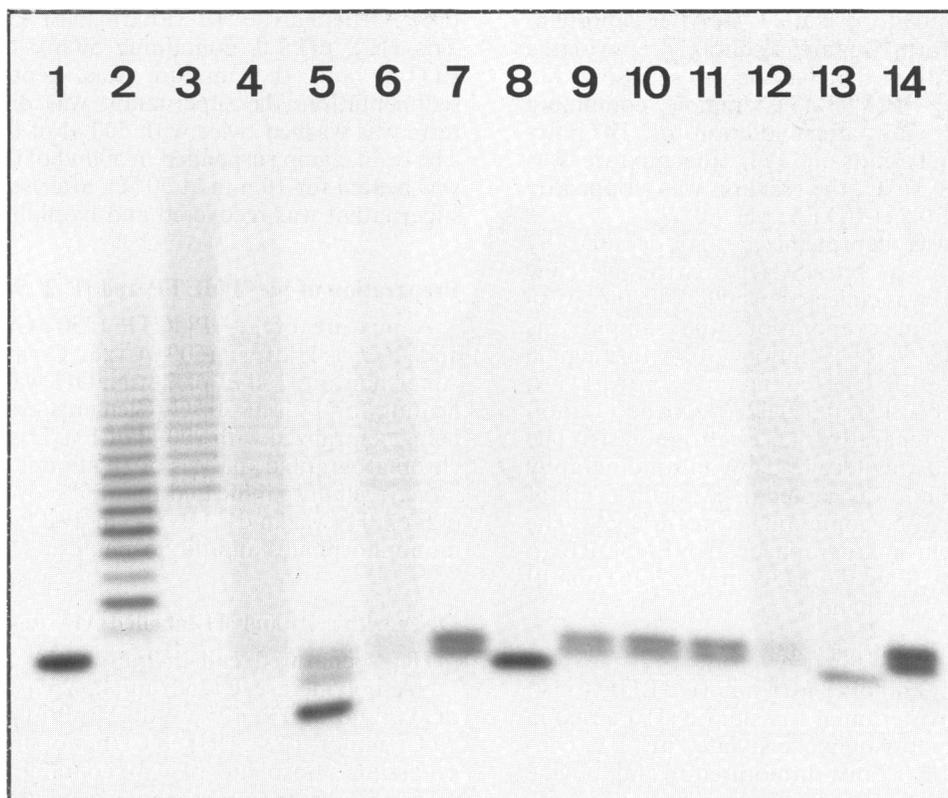
Heat-denatured calf-thymus DNA (2.5 mg), *E. coli* DNA polymerase I (250 units), 25  $\mu$ M each of dATP, dGTP and dTTP, 125  $\mu$ M-dCTP, 0.14  $\mu$ M-dUTP ( $\alpha\text{-}^{32}\text{P}$ , 29 Ci/mmol;  $\text{1}'\text{,2}'\text{,5-}^3\text{H}$ , 58 Ci/mmol) in 60 ml of 67 mM-potassium phosphate, pH 7.4, containing 6.7 mM- $\text{MgCl}_2$  and 1 mM-2-mercaptoethanol, were incubated for 10 h at 22 °C; the reaction was stopped with 4 ml of 0.2 M-EDTA.

The solution was dialysed against 30 mM-sodium acetate/acetic acid, pH 4.5, containing 0.1 M-NaCl and 1 mM- $\text{ZnCl}_2$ , before addition of S1 nuclease followed by a 20 min incubation at 30 °C to degrade the non-radioactive DNA template that remained single-stranded; the reaction was stopped with 2 ml of 0.2 M-EDTA.

The reaction mixture was deproteinized with chloroform/isoamyl alcohol, dialysed extensively against 5 mM-potassium phosphate, pH 6.8, then chromatographed on a hydroxyapatite column (0.9 cm  $\times$  16 cm) equilibrated with the same buffer. Elution was carried out in three steps using 16 ml portions of potassium phosphate, pH 6.8, of increasing concentrations (100, 150 and 300 mM); the two first steps eliminated the single-stranded DNA and its degradation products, whereas the  $^{32}\text{P}$ - and  $^3\text{H}$ -labelled double-stranded DNA was collected in the 300 mM solution.

After dialysis against 50 mM-Hepes/KOH, pH 8.0, containing 1 mM-EDTA and 1 mM-2-mercaptoethanol, uracil-DNA glycosylase was added and the solution was incubated at 37 °C. Release of  $[\text{5-}^3\text{H}]\text{uracil}$  was followed by measurements, on aliquots, of the radioactivity soluble in 5%  $\text{HClO}_4$ ; this acid-soluble  $^3\text{H}$  reached a maximum of 36.8% after 10 min whereas the acid-soluble  $^{32}\text{P}$  was only 0.3%. The incubation with the enzyme was stopped after 1 h, the solution was deproteinized with chloroform/isoamyl alcohol, then extensively dialysed against 50 mM-Hepes/KOH (pH 8.0)/1 mM-EDTA.

The AP sites in the double-stranded DNA were thus labelled with  $^{32}\text{P}$  on their 5' side and with  $^3\text{H}$  in the 1' and 2' positions of the base-free deoxyribose. The specific radioactivities were respectively 29 Ci/mmol for the 5'-phosphate and 37 Ci/mmol for the base-free deoxyribose.



**Fig. 2. Comparative actions of alkaline pH, *E. coli* endonuclease IV or *E. coli* endonuclease III on an AP site-containing double-stranded oligonucleotide: action of T4 DNA polymerase 3'-5' exonuclease on the endonuclease III cleavage products**

[5'-<sup>32</sup>P]pdT<sub>8</sub>dA (lane 1) was extended into [5'-<sup>32</sup>P]pdT<sub>8</sub>dAdT<sub>n</sub> (lane 2); poly(A)-Sepharose chromatography enabled the isolation only of polymers [5'-<sup>32</sup>P]pdT<sub>8</sub>dAdT<sub>n</sub> having more than 15 nucleotides (lane 3). These polymers were depurinated to give [5'-<sup>32</sup>P]pdT<sub>8</sub>d(-)dT<sub>n</sub> (lane 4). [5'-<sup>32</sup>P]pdT<sub>8</sub>d(-)dT<sub>n</sub> (0.5 nmol; 1 μCi) and pdA<sub>40-60</sub> (1.5 nmol) were dissolved in 200 μl of 1 mM-EDTA. Of this solution, 10 μl was mixed with an equal volume of 0.1 M-NaOH, warmed at 37 °C for 2 h, and neutralized (lane 5). To another 27 μl was added 3 μl of 0.5 M-Hepes/KOH, pH 8.2, containing 2 M-NaCl, 10 mM-EDTA and 10 mM-2-mercaptoethanol. A 10 μl control was taken and incubated without enzyme for 60 min at 37 °C (lane 12). The remaining 20 μl were supplemented with *E. coli* endonuclease IV and divided in two parts which were incubated at 37 °C for 10 min (lane 13) or 60 min (lane 8). To the rest of the [5'-<sup>32</sup>P]pdT<sub>8</sub>d(-)dT<sub>n</sub> solution (162 μl) was added 18 μl of 0.5 M-Hepes/KOH, pH 7.8, containing 1 M-KCl, 10 mM-EDTA and 10 mM-dithiothreitol. Of this, 10 μl were kept as a control that was incubated for 60 min at 37 °C (lane 6). The remaining 170 μl were supplemented with endonuclease III and incubated at 37 °C; 10 μl aliquots were taken after 40 min (lane 7) and at the end of the 60 min incubation (lane 14). The solution was then deproteinized with chloroform/isoamyl alcohol and the pdA<sub>40-60</sub> was removed with poly(A)-Sepharose as described in the Materials and methods section. The remaining radioactive oligonucleotide was redissolved in 27 μl of water to which 3 μl of 500 mM-Hepes/KOH, pH 8.0, containing 100 mM-MgCl<sub>2</sub> and 10 mM-2-mercaptoethanol was added. An aliquot (10 μl) was taken and incubated for 60 min at 37 °C without enzyme (lane 9). The remaining solution was supplemented with T4 DNA polymerase (10 units) and divided in 10 μl portions that were incubated at 37 °C for 5 min (lane 10) or 60 min (lane 11). Analysis on polyacrylamide gel was followed by autoradiography.

#### DEAE-Sephadex chromatography

After addition of deoxyribose 5-phosphate and dUMP as markers, the samples, 10-fold diluted with 0.1 M-borate/HCl, pH 8.3, were placed on a 0.9 cm × 30 cm column of DEAE-Sephadex A25 previously equilibrated with the same borate buffer. The elution was carried out with 200 ml of a linear 0-0.25 M-NaCl gradient, then with 2 M-NaCl in borate buffer; 3 ml fractions were collected. The elution peak of dUMP was located by absorbance measurements at 260 nm; deoxyribose 5-phosphate was identified by the diphenylamine reaction followed by absorbance measurements at 600 nm. A 100 μl aliquot was taken from each fraction for <sup>3</sup>H and <sup>32</sup>P radioactivity determinations.

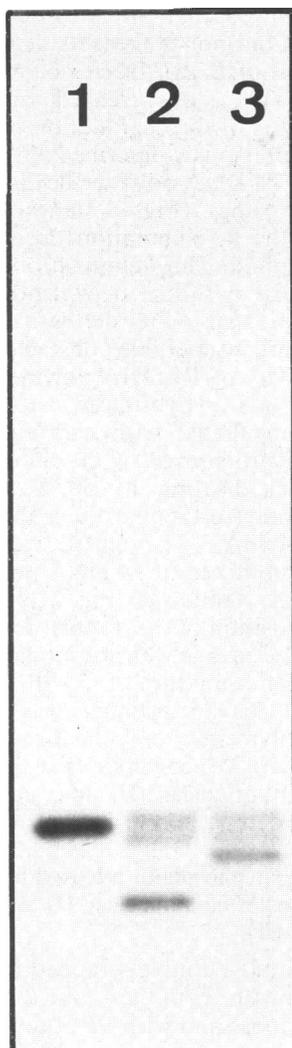
#### RESULTS

##### Nicking [5'-<sup>32</sup>P]pdT<sub>8</sub>d(-)dT<sub>n</sub> with *E. coli* endonuclease IV, alkaline pH or *E. coli* endonuclease III

The size of [5'-<sup>32</sup>P]pdT<sub>8</sub>d(-)dT<sub>n</sub> was between 15 and 60 nucleotides (Fig. 2, lane 4). It was hybridized with poly(dA)<sub>40-60</sub> in a nucleotide ratio of 1:2.

This double-stranded polymer was first exposed to *E. coli* endonuclease IV, then analysed by denaturing gel electrophoresis followed by autoradiography (Fig. 2, lanes 8 and 13). Endonuclease IV is a 5' AP endonuclease; it had thus left [5'-<sup>32</sup>P]pdT<sub>8</sub>.

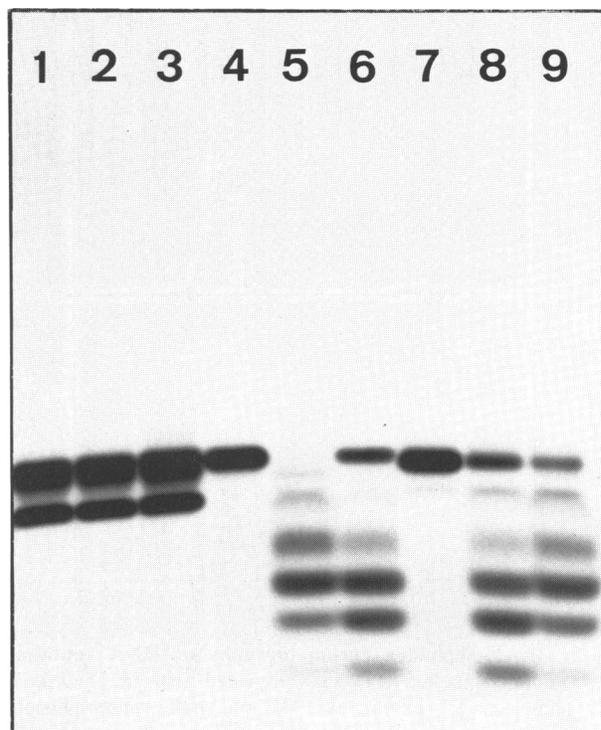
The double-stranded polymer was also incubated in 0.05 mM-NaOH for 2 h at 37 °C before being analysed.



**Fig. 3.** Alkaline degradation of  $[5'-^{32}\text{P}]\text{pdT}_8\text{d}(-)\text{dT}_n$

$[5'-^{32}\text{P}]\text{pdT}_8\text{d}(-)\text{dT}_n$  (31 pmol; 0.062  $\mu\text{Ci}$ ) in 16  $\mu\text{l}$  of 0.05 M-NaOH was incubated for 2 h at 37  $^\circ\text{C}$ ; the solution was then neutralized with 2  $\mu\text{l}$  of 0.4 M-HCl. Then 2  $\mu\text{l}$  of 1 M-imidazole/HCl, pH 6.0, containing 0.1 M- $\text{MgCl}_2$  and 10 mM-2-mercaptoethanol was added, followed by 1  $\mu\text{l}$  of 50 mM-ATP, and an aliquot (7  $\mu\text{l}$ ) was incubated for 15 min at 37  $^\circ\text{C}$  without enzyme (lane 2). The remaining solution was supplemented with a 3'-phosphatase (T4 polynucleotide kinase; 5 units) and incubated at 37  $^\circ\text{C}$  for 5 min (lane 3). Analysis on polyacrylamide gel was followed by autoradiography;  $[5'-^{32}\text{P}]\text{pdT}_8\text{dA}$  (lane 1) was used as standard.

The autoradiography (Fig. 2, lane 5; Fig. 3, lane 2) shows three rungs: a doublet which is at the level of  $[5'-^{32}\text{P}]\text{pdT}_8\text{dA}$  (Fig. 2, lane 1; Fig. 3, lane 1), indicating a cleavage 3' to the AP site which is very likely the result of a  $\beta$ -elimination; the third rung, further down, is the result of some  $\delta$ -elimination yielding  $[5'-^{32}\text{P}]\text{pdT}_8\text{p}$  with a 3' phosphate end (Grossman & Grafstrom, 1982). This was verified in two ways: the third rung was the only one seen after a more drastic alkaline treatment (0.2 M-NaOH, 30 min at 65  $^\circ\text{C}$ ) (not shown); following a treatment with T4 polynucleotide kinase, which has a 3'

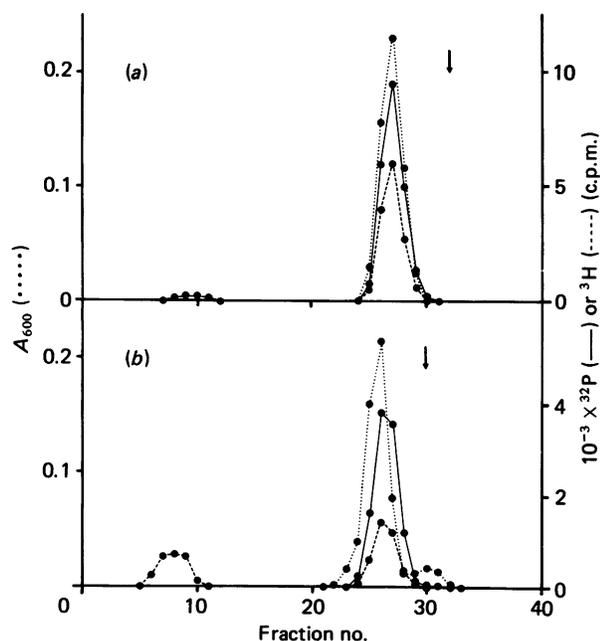


**Fig. 4.** Action of T4 DNA polymerase 3'-5' exonuclease on  $\text{pdT}_8\text{dA}$ ,  $\text{pdT}_8\text{d}(-)$  and the alkaline degradation product of  $\text{pdT}_8\text{d}(-)\text{dT}_n$

$[5'-^{32}\text{P}]\text{pdT}_8\text{dA}$  (32.5 pmol) was dissolved in 30  $\mu\text{l}$  of 8.3 mM-NaCl. Another sample of  $[5'-^{32}\text{P}]\text{pdT}_8\text{dA}$  (32.5 pmol) in 25  $\mu\text{l}$  of 10 mM-HCl was heated for 1 h at 65  $^\circ\text{C}$  to yield  $[5'-^{32}\text{P}]\text{pdT}_8\text{d}(-)$ , before addition of 5  $\mu\text{l}$  of 50 mM-NaOH.  $[5'-^{32}\text{P}]\text{pdT}_8\text{d}(-)\text{dT}_n$  (77 pmol) in 40  $\mu\text{l}$  of 0.05 M-NaOH was incubated for 2 h at 37  $^\circ\text{C}$ ; the solution was then neutralized with 2  $\mu\text{l}$  of 1 M-HCl. To the solution of  $[5'-^{32}\text{P}]\text{pdT}_8\text{dA}$  (lanes 4, 5 and 6),  $[5'-^{32}\text{P}]\text{pdT}_8\text{d}(-)$  (lanes 7, 8 and 9) or alkali-degraded  $[5'-^{32}\text{P}]\text{pdT}_8\text{d}(-)\text{dT}_n$  (lanes 1, 2 and 3) (30  $\mu\text{l}$  each) was added 3  $\mu\text{l}$  of 200 mM-Hepes/KOH, pH 8.0, containing 100 mM- $\text{MgCl}_2$  and 10 mM-2-mercaptoethanol. An aliquot (10  $\mu\text{l}$ ) was taken from each solution and incubated for 60 min at 37  $^\circ\text{C}$  without enzyme (lanes 1, 4 and 7). The remaining solutions were supplemented with T4 DNA polymerase (2 units) and divided in two parts incubated at 37  $^\circ\text{C}$  for 5 min (lanes 2, 5 and 8) or 60 min (lanes 3, 6 and 9). Analysis on polyacrylamide gel was followed by autoradiography.

phosphatase activity, the third rung disappeared and was replaced by a rung closer to the doublet (Fig. 3, lane 3) at the position of  $[5'-^{32}\text{P}]\text{pdT}_8$ .

When the double-stranded polymer was incubated with endonuclease III, the analysis showed a doublet at the level of  $[5'-^{32}\text{P}]\text{pdT}_8\text{dA}$  (Fig. 2, lanes 7 and 14), one step above  $[5'-^{32}\text{P}]\text{pdT}_8$  (Fig. 2, lanes 8 and 13). It is thus the phosphodiester bond which is the immediate neighbour of the AP site on its 3' side which is broken by endonuclease III, as many authors observed before us, but what is new is that the nicking yields two different entities giving a doublet on the electrophoretogram exactly in the position of the doublet obtained by an alkaline  $\beta$ -elimination.



**Fig. 5.** DEAE-Sephadex chromatography of DNA containing doubly-labelled AP sites digested with *E. coli* endonuclease VI (exonuclease III) and snake venom phosphodiesterase or with *E. coli* endonuclease III and endonuclease VI (exonuclease III)

(a) DNA containing  $[5'-^{32}\text{P};1',2'-^3\text{H}]$ AP sites ( $38\ \mu\text{g}$ ) in  $1.7\ \text{ml}$  of  $12.5\ \text{mM}$ -Hepes/KOH, pH 8.0, was supplemented with  $18\ \mu\text{l}$  of  $1\ \text{M}$ - $\text{MgCl}_2$ ,  $1\ \mu\text{l}$  of *E. coli* endonuclease VI (exonuclease III; 25 units) solution and  $60\ \mu\text{l}$  of snake venom phosphodiesterase (0.004 unit) solution, and incubated for 90 min at  $37^\circ\text{C}$ . The reaction was stopped with  $180\ \mu\text{l}$  of  $0.2\ \text{M}$ -EDTA. (b) DNA containing  $[5'-^{32}\text{P};1',2'-^3\text{H}]$ AP sites ( $17\ \mu\text{g}$ ) in  $760\ \mu\text{l}$  of  $60\ \text{mM}$ -Hepes/KOH, pH 7.8, containing  $0.1\ \text{M}$ -KCl,  $1\ \text{mM}$ -EDTA,  $1\ \text{mM}$ -2-mercaptoethanol and  $40\ \mu\text{l}$  of *E. coli* endonuclease III solution was incubated for 3 h at  $37^\circ\text{C}$ . After addition of  $8\ \mu\text{l}$  of  $1\ \text{M}$ - $\text{MgCl}_2$  and  $1\ \mu\text{l}$  of *E. coli* endonuclease VI (exonuclease III; 25 units) solution, the incubation was continued for 30 min at the same temperature. The reaction was stopped with  $80\ \mu\text{l}$  of  $0.2\ \text{M}$ -EDTA. After addition of dUMP and deoxyribose 5-phosphate as elution markers, the samples were analysed on DEAE-Sephadex as described in the Materials and methods section. The  $^{32}\text{P}$  (—) and  $^3\text{H}$  (----) radioactivities were measured on the collected fractions. The dUMP peak is indicated by an arrow; the deoxyribose 5-phosphate was measured at  $A_{600}$  (.....) after reaction with diphenylamine.

#### Action of the 3'-5' exonuclease of T4 DNA polymerase on the cleavage product, by endonuclease III, of $[5'-^{32}\text{P}]$ pdT<sub>8</sub>d(-)dT<sub>n</sub> hybridized to poly(dA)

$[5'-^{32}\text{P}]$ pdT<sub>8</sub>dA and  $[5'-^{32}\text{P}]$ pdT<sub>8</sub>d(-) were submitted to T4 DNA polymerase in  $0.02\ \text{M}$ -Hepes/KOH, pH 8.0, containing  $10\ \text{mM}$ - $\text{MgCl}_2$  and  $1\ \text{mM}$ -2-mercaptoethanol, then analysed by gel electrophoresis. As expected,  $[5'-^{32}\text{P}]$ pdT<sub>8</sub>dA was degraded by the 3'-5' exonuclease activity of the enzyme (Fig. 4, lanes 5 and 6); but  $[5'-^{32}\text{P}]$ pdT<sub>8</sub>d(-) was also degraded (Fig. 4, lanes 8 and 9) showing that a base-free deoxyribose at the 3' end of the oligonucleotide was not an obstacle to this activity. This is in agreement with a previous result (Bailly & Verly,

1984) which showed that an AP site within a polynucleotide chain did not prevent its degradation by the Klenow fragment of *E. coli* DNA polymerase I.

$[5'-^{32}\text{P}]$ pdT<sub>8</sub>d(-)dT<sub>n</sub> was cleaved by mild alkaline treatment; analysis of the degradation products showed the three rungs previously described (Fig. 4, lane 1). An incubation with T4 DNA polymerase did not change the position of the rungs (Fig. 4, lanes 2 and 3). The insensitivity of the  $\beta\delta$ -elimination product is due to a terminal 3'-phosphate. The  $\beta$ -elimination products do not have a 3'-terminal base-free deoxyribose but rather a 2',3'-unsaturated sugar or its derivative (Fig. 1). Such a 3' end is an insuperable obstacle to the 3'-5' exonuclease activity of T4 DNA polymerase.

$[5'-^{32}\text{P}]$ pdT<sub>8</sub>d(-)dT<sub>n</sub>, hybridized with an excess of poly(dA), was incubated with endonuclease III. The solution was deproteinized with chloroform/isoamyl alcohol; a quick heating at  $50^\circ\text{C}$  followed by a treatment with poly(A)-Sephadex enabled the elimination of the poly(dA). The eluted radioactive oligonucleotide was submitted to T4 DNA polymerase before analysis on polyacrylamide gel. Fig. 2 (lanes 9, 10 and 11) shows that the position of the doublet resulting from the endonuclease III cleavage was not modified by T4 DNA polymerase. When a mixture of  $[5'-^{32}\text{P}]$ pdT<sub>8</sub>dA and the endonuclease III cleavage products was treated together with T4 DNA polymerase, only the former was degraded (results not shown). We conclude that the cleavage 3' to the AP site by endonuclease III does not leave a 3' end with a base-free deoxyribose.

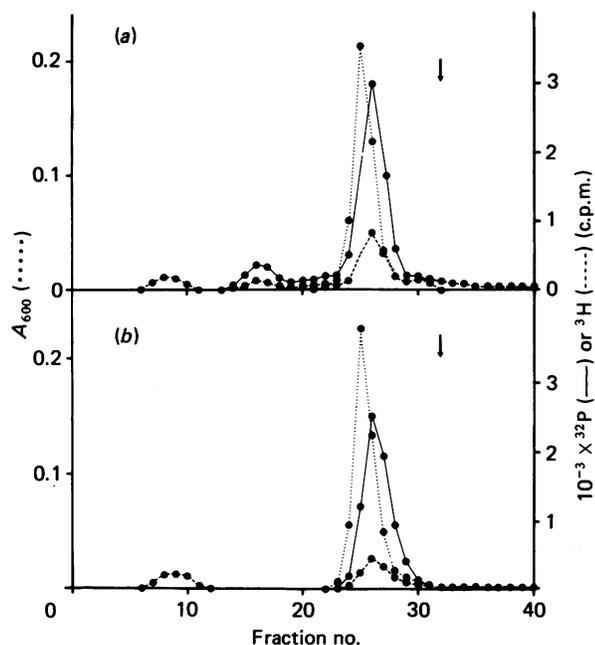
#### Nature of the sugar phosphate released by the conjugated actions of endonuclease III and endonuclease VI (exonuclease III)

We have prepared a double-stranded DNA containing AP sites labelled with  $^3\text{H}$  in the 1' and 2' positions of the base-free deoxyribose and with  $^{32}\text{P}$  5' to this deoxyribose (see the Materials and methods section). To be able to analyse the action of endonuclease III, we shall first compare the results of 3' nicks by hydrolysis and by  $\beta$ -elimination.

**Hydrolysis.** The DNA containing the doubly-labelled AP sites was digested with *E. coli* endonuclease VI (exonuclease III) and snake venom phosphodiesterase (Fig. 1). The digestion product was analysed on DEAE-Sephadex as described in the Materials and methods section. The results are in Fig. 5(a).

It can be seen that 92% of the  $^3\text{H}$  and 98% of the  $^{32}\text{P}$  put on the column were recovered in a single peak coincident with the deoxyribose 5-phosphate marker. The  $^3\text{H}/^{32}\text{P}$  ratio in the eluted  $[1,2-^3\text{H}]$ deoxyribose 5- $[^{32}\text{P}]$ phosphate was thus practically identical with that of the AP-site-containing DNA substrate. A small  $^3\text{H}$  peak emerged from the column before the application of the gradient; this  $^3\text{H}$ , which was not volatile, was probably in  $[1,2-^3\text{H}]$ deoxyribose molecules since the snake venom phosphodiesterase was still slightly contaminated with a 5'-nucleotidase.

**$\beta$ -Elimination.** The DNA containing doubly-labelled AP sites was incubated at pH 11.5 for 6 h at  $37^\circ\text{C}$ . After neutralization and addition of  $\text{MgCl}_2$  and *E. coli* endonuclease VI (exonuclease III), the incubation at  $37^\circ\text{C}$  was continued for 30 min. The sample was



**Fig. 6.** DEAE-Sephadex chromatography of DNA containing doubly-labelled AP sites treated at alkaline pH or with Lys-Trp-Lys, and subsequently digested with *E. coli* endonuclease VI (exonuclease III)

(a) To DNA (36  $\mu$ g) containing [5'- $^{32}$ P;1',2'- $^3$ H]AP sites in 800  $\mu$ l of 25 mM-sodium cacodylate, pH 6.5 (adjusted with HCl) containing 1 mM-EDTA was added 4  $\mu$ l of 0.2 M-Lys-Trp-Lys and the mixture was incubated for 3 h at 37  $^{\circ}$ C. The solution was then supplemented with 9  $\mu$ l of 1 M-MgCl<sub>2</sub> and 1  $\mu$ l of *E. coli* endonuclease VI (exonuclease III; 25 units) solution and the incubation was continued at 37  $^{\circ}$ C for 30 min. The reaction was stopped with 80  $\mu$ l of 0.2 M-EDTA. (b) DNA (24  $\mu$ g) containing [5'- $^{32}$ P;1',2'- $^3$ H]AP sites in 600  $\mu$ l of 0.1 M-potassium phosphate, pH 11.5, containing 10 mM-EDTA was incubated for 6 h at 37  $^{\circ}$ C; the solution was then neutralized with 28  $\mu$ l of 1 M-HCl. After addition of 7  $\mu$ l of 1 M-MgCl<sub>2</sub> and 1  $\mu$ l of *E. coli* endonuclease VI (exonuclease III; 25 units) solution, it was further incubated at 37  $^{\circ}$ C for 30 min. The reaction was stopped with 65  $\mu$ l of 0.2 M-EDTA. The samples were chromatographed on DEAE-Sephadex. Markers and symbols are as in Fig. 5.

then analysed on DEAE-Sephadex. The results are in Fig. 6(b).

25% of  $^3$ H and 77% of  $^{32}$ P placed on the column were eluted in a single peak delayed by one fraction from the peak of the deoxyribose 5-phosphate marker. Some  $^3$ H came out of the column before the application of the ionic gradient; 92% of it was volatile. The double-labelled sugar phosphate eluted just after the deoxyribose 5-phosphate marker is its 2,3-unsaturated derivative. The subtraction of a H<sup>+</sup> from the 2' position of the base-free sugar during the  $\beta$ -elimination reaction explains the appearance of volatile  $^3$ H and also the decreased  $^3$ H/ $^{32}$ P ratio in the isolated sugar phosphate. But the reaction in alkaline medium is complicated by a partial  $\delta$ -elimination (Fig. 1). For this reason, the experiment was repeated with Lys-Trp-Lys as a  $\beta$ -elimination catalyst (Pierre & Laval, 1981; Behmoaras *et al.*, 1981).

The DNA containing doubly-labelled AP sites was incubated for 3 h at 37  $^{\circ}$ C with 1 mM-Lys-Trp-Lys in

cacodylate buffer, pH 6.5. At this time, some AP sites were still intact. After addition of MgCl<sub>2</sub>, the sample was incubated with *E. coli* endonuclease VI (exonuclease III) for 30 min at 37  $^{\circ}$ C. The sample was chromatographed on DEAE-Sephadex. The results are in Fig. 6(a). 27% of the  $^3$ H and 63% of the  $^{32}$ P put on the column were eluted in a single peak delayed by one fraction with respect to the deoxyribose 5-phosphate marker peak. Some  $^3$ H, most of it volatile, came out of the column before application of the ionic gradient, and the  $^3$ H/ $^{32}$ P ratio had been reduced in the eluted sugar phosphate. An intermediary peak in fractions 13–17 might be due to the formation of a Schiff base between the tripeptide and the sugar phosphate.

***E. coli* endonuclease III.** The DNA containing doubly-labelled AP sites was incubated with *E. coli* endonuclease III at 37  $^{\circ}$ C; after 1 h the  $^{32}$ P radioactivity soluble in 5% HClO<sub>4</sub> had reached a maximum, showing that the enzyme had nicked the DNA strands near all the AP sites. After addition of MgCl<sub>2</sub> and *E. coli* endonuclease VI (exonuclease III), the incubation was continued at 37  $^{\circ}$ C for 30 min; at this time, the  $^3$ H and  $^{32}$ P radioactivities non-adsorbable on Norit were 100%. After addition of EDTA, the sample was chromatographed on DEAE-Sephadex. The results are in Fig. 5(b). 57% of the  $^3$ H and 96% of the  $^{32}$ P put on the column were eluted in a single peak delayed by one fraction from the peak of the deoxyribose 5-phosphate marker. The  $^3$ H/ $^{32}$ P ratio had thus been reduced in the sugar phosphate; moreover, some  $^3$ H, 100% of it volatile, was eluted from the column before application of the ionic gradient.

The nicking 3' to AP sites by *E. coli* endonuclease III is thus not a hydrolysis; it is much more like a  $\beta$ -elimination (Fig. 1). Indeed, the nicking is associated with a loss of  $^3$ H, very likely from the 2' position of the base-free deoxyribose, which appears as volatile  $^3$ H (probably  $^3$ H<sub>2</sub>O). Moreover, after nicking 5' to the AP sites with *E. coli* endonuclease VI (exonuclease III), one gets a sugar phosphate with the expected decreased  $^3$ H/ $^{32}$ P ratio, which behaves chromatographically as the product obtained by the conjugated actions of  $\beta$ -elimination and 5' hydrolysis.

## DISCUSSION

We have synthesized an oligonucleotide containing an AP site and labelled with  $^{32}$ P at its 5' end: [5'- $^{32}$ P]pdT<sub>8</sub>d(-)dT<sub>n</sub>. Mild alkaline treatment of this oligonucleotide followed by gel electrophoresis yielded three bands. The faster moving one was identified as [5'- $^{32}$ P]pdT<sub>8</sub>p produced by  $\beta\delta$ -elimination. The two others, which are very close to one another forming a doublet migrating at the level of [5'- $^{32}$ P]pdT<sub>8</sub>dA, appear to be related to the  $\beta$ -elimination process: one could be the primary product of  $\beta$ -elimination, bearing a 2',3'-unsaturated derivative of deoxyribose at its 3' end; the other could be terminated by the derived 2-oxocyclopent-1-enyl (Jones *et al.*, 1968) (Fig. 1). Whatever the chemical nature of the 3' end created by  $\beta$ -elimination, in contrast with a 3'-terminal base-free deoxyribose, it prevents the degradation of the oligonucleotide by the 3'-5' exonuclease activity of T4 DNA polymerase.

When  $[5'\text{-}^{32}\text{P}]\text{pdT}_8\text{d}(-)\text{dT}_n$  was cleaved with *E. coli* endonuclease III and the cleavage product was analysed by gel electrophoresis, a doublet at the level of  $[5'\text{-}^{32}\text{P}]\text{pdT}_8\text{dA}$ , identical with the one obtained by alkaline  $\beta$  elimination, was observed. Moreover, the cleavage products could not be degraded by the 3'-5' exonuclease activity of T4 DNA polymerase. These results show that the cleavage 3' to the AP sites, by endonuclease III, cannot be a hydrolysis leaving 3'-OH and 5'-phosphate ends. It is more likely the result of a  $\beta$ -elimination reaction.

We have also synthesized DNA containing doubly-labelled AP sites: with  $^3\text{H}$  in the 1' and 2' positions of the base-free deoxyribose and with  $^{32}\text{P}$  5' to this deoxyribose. A complete degradation of this DNA with a mixture of endonuclease VI (exonuclease III) and snake venom phosphodiesterase yielded deoxyribose 5-phosphate with the same  $^3\text{H}/^{32}\text{P}$  ratio as the substrate DNA. On the other hand, a combined excision by alkaline  $\beta$ -elimination and endonuclease VI (exonuclease III) hydrolysis resulted in a loss of  $^3\text{H}$  (from the 2' position of the base-free deoxyribose) and the formation of a sugar phosphate, different from deoxyribose 5-phosphate, with a lower  $^3\text{H}/^{32}\text{P}$  ratio than the DNA substrate. Utilization of the tripeptide Lys-Trp-Lys for the nicking 3' to the AP site yielded the same result, showing that this nicking is also the result of a  $\beta$ -elimination (Fig. 1).

Treatment of the DNA containing doubly-labelled AP sites by *E. coli* endonuclease III also led to a loss of  $^3\text{H}$ , and a subsequent treatment with *E. coli* endonuclease VI (exonuclease III) gave a sugar phosphate with a lower  $^3\text{H}/^{32}\text{P}$  ratio than the DNA substrate, that behaved chromatographically as the  $\beta$ -elimination/endonuclease VI (exonuclease III) excision product and not as deoxyribose 5-phosphate.

From these results, we can be sure that the action of *E. coli* endonuclease III on AP site-containing DNA is not the hydrolysis of the phosphodiester bond 3' to the AP sites leaving 3'-OH and 5'-phosphate ends. The nicking 3' to the AP sites, which gives 5'-phosphate ends, is rather a  $\beta$ -elimination reaction leaving a 2',3'-unsaturated deoxyribose as a primary product that can evolve subsequently in a way similar to that observed after an alkaline  $\beta$ -elimination (Fig. 1). This raises the question whether it is reasonable to call this enzyme

'endonuclease III'; it is a DNA glycosylase and a phosphoric monoester-lyase (to be placed in EC class 4), but not an endonuclease (which is a phosphoric diester hydrolase, EC 3.1.4.-).

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